TRANSDUCTION IN *BACILLUS SUBTILIS*

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ABSTRACT

THORNE, CURTIS B. (Fort Detrick, Frederick, Md.). Transduction in *Bacillus subtilis*. J. Bacteriol. **83**:106–111. 1962.—A bacteriophage, SP-10, isolated from soil carries out general transduction in *Bacillus subtilis*. Phage propagated on a streptomycin-resistant mutant of the wild-type strain W-23 was capable of transducing to prototrophy strain 168 (indole$^-$), as well as all of the auxotrophic mutants of W-23-S^r tested, which included mutants requiring arginine, histidine, adenine, guanine, thiamine, leucine, or methionine. Although strain 168 was transduced by phage SP-10, lytic activity on this strain could not be detected and attempts to propagate the phage on it failed. Transductions occurred at frequencies in the range of 10^{-6} to 10^{-5} per plaque-forming unit. Homologous phage was ineffective, deoxyribonuclease had no effect on the frequency of transduction, and transduction was prevented by the addition of phage antiserum. Phage SP-10 was capable of lysogenizing strain W-23-S^r, and this condition was maintained through repeated growth and sporulation cycles in potato-extract medium. Although heating at 65 C for 60 min inactivated free phage particles, spores retained their lysogenic condition after such heat treatment. When heat-treated spores of the lysogenic cultures were used as inocula for growth in a nutrient broth-yeast extract-glucose medium, filtrates contained 10^9, or more, phage particles per ml.

Although transduction, phage-mediated genetic transfer, has been reported to occur in several species of bacteria (Hartman and Goodgal, 1959), until recently (Thorne, 1961) there had been no report dealing with this type of genetic transfer in *Bacillus subtilis*. This report presents details of experiments on a phage isolated from soil, which participates in general transduction in *B. subtilis*.

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MATERIALS AND METHODS

Bacterial strains. B. subtilis strains W-23-S^r (a streptomycin-resistant mutant of wild-type W-23) and 168 (indole$^-$) were obtained from Maurice Fox. Although strain W-23-S^r was streptomycin-resistant, I did not study this characteristic in these experiments. The strain of *B. licheniformis* was ATCC 9945A. Auxotrophic mutants of *B. subtilis* W-23-S^r and *B. licheniformis* were isolated by the method of Iyer (1960) after irradiation with ultraviolet light.

Media and cultural conditions. NBY medium was composed of 8 g nutrient broth (Difco Laboratories, Detroit, Mich.) and 3 g Difco yeast extract per liter. NBYG medium was NBY with 1 g glucose per liter. Minimal medium was composed of the following in g/liter: $(NH_4)_2SO_4$, 2; KH_2PO_4, 6; K_2HPO_4, 14; sodium citrate, 1; glucose, 5 (autoclaved separately); $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.04; $MnSO_4 \cdot H_2O$, 0.00025; pH 6.9 to 7.0. For most of the solid media, 25 g agar/liter was added. L-Glutamic acid (1 g/liter) was usually added to the minimal agar to allow colonies to develop faster. Distilled water was used in all media.

Cells for transduction were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium. NBY broth was used for strain 168 and minimal broth plus yeast extract (3 g/liter) was used for mutants of W-23-S^r. Flasks were inoculated with 5 ml of a 16-hr culture and incubated on a shaker for 5 hr at 37 C. Viable counts were determined by plating on NBY agar.

Spores were produced in 1-liter shaken flasks containing 90 ml of potato extract medium prepared as follows: Diced potatoes (100 g) were boiled 5 min in 500 ml of water, and the material was filtered through Whatman no. 1 paper on a Büchner funnel; 1 g Difco yeast extract and 10 g N-Z Case peptone (Sheffield

Chemical, Norwich, N. Y.) were added, the pH was adjusted to 7.2 with NaOH, and the solution was diluted to 1 liter. Cultures were incubated 72 hr at 37 C. Spores were collected by centrifugation, washed twice with water, held 30 min at 65 C, and stored at 5 C.

Isolation of phages. Both *B. subtilis* W-23-S^r and *B. licheniformis* were used as indicator strains in isolating phages from soil. Samples of the soils were added to 250-ml flasks containing 50 ml of NBY broth; the flasks were then incubated on a shaker at 37 C. After 2 hr, 1 ml of a 16-hr NBY broth culture of the appropriate indicator strain was added to each flask and incubation was continued for about 16 hr. The cultures were centrifuged and filtered through fritted glass to remove bacteria, and filtrates were tested for plaque formation against each of the two indicator strains by the soft agar overlay method. Single plaques were transferred into NBY broth inoculated with spores of the respective indicator strains. After incubation for 12 to 16 hr, filtrates from these were used for further phage propagation.

Phage propagation. Phage was propagated on the appropriate organism either in shaken flasks of NBYG or on NBY agar overlayered with a medium containing Difco peptone, 10 g/liter, and agar, 7 g/liter. When liquid medium was used, the cells were removed by centrifugation and filtration through ultrafine fritted glass filters. When the soft agar technique was used, the phage lysate was harvested in peptone diluent, centrifuged, and filtered. Tests indicated that little or no phage was lost by filtering through glass filters. Phage from lysogenic strains was obtained routinely by inoculating about 1×10^7 spores into shaken flasks of NBYG broth and incubating for 12 to 16 hr.

Assay of phage. For titrating phage suspensions and for determining plaque morphology, I used the agar layer technique (Adams, 1959). The bottom layer of agar (25 ml) was Difco dehydrated nutrient agar (23 g/liter), with the following added (in g/liter): NaCl, 5; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.05; $CaCl_2 \cdot 2H_2O$, 0.15. Soft agar for overlayering contained nutrient broth (8 g/liter), agar (7 g/liter), and the same salts as were used in the bottom layer. In early tests, NBY agar was used for phage assays, but in the course of these experiments plaque formation suddenly became erratic. Several media were tested and best results were obtained with the one described. The difficulty experienced with NBY agar has not been explained, but it might be a result of variation in different lots of medium constituents or in distilled water. The new medium has consistently given good results.

Difco peptone (10 g/liter) was the standard diluent for phage suspensions; 0.1 ml of the appropriate dilution of phage and 0.1 ml of a suspension containing approximately 1×10^9 spores of the indicator strain per ml were mixed with 3 ml of soft agar and poured onto the base agar. Plaques were counted after 16 to 24 hr at 37 C, and phage titers expressed as plaque-forming units (PFU) per ml. Plates of agar base were prepared the same day they were used, since erratic results were obtained with stored plates. Unglazed porcelain covers were used on the plates, to eliminate smearing of plaques caused by excessive moisture.

Phage antiserum. Antiserum was prepared in rabbits by intravenous injection of phage SP-10, which had been collected by high speed centrifugation and washed twice with a solution containing 0.15 M NaCl and 0.001 M $MgSO_4$. Each of three rabbits was injected with 0.5 ml of suspension containing about 1×10^9 PFU/ml, and injections were repeated after 2 and 9 days. Sera were collected and pooled a week after the last injection.

Deoxyribonucleic acid (DNA). Crude DNA was prepared from strain W-23-S^r, as described by Ephrati-Elizur, Srinivason, and Zamenhof (1961). No attempt was made to purify the DNA, and quantitative determinations were not made.

Deoxyribonuclease. This was a $1 \times$ crystallized product of Worthington Biochemical Corp. (Freehold, N. J.).

RESULTS

Phages isolated. Several temperate and virulent phages were isolated. Some of them were active on both *B. subtilis* and *B. licheniformis*, and others were active only on *B. licheniformis*. One of the phages (designated SP-10), which is temperate for *B. subtilis*, was selected for further study and experiments on transduction.

Transduction of strain 168. The results of a transduction experiment are shown in Table 1. Phage SP-10 was propagated on strain W-23-S^r

and used to transduce strain 168 (indole$^-$). No spontaneous reversion occurred, since no colonies grew from the control vial which contained cells without phage. Similarly, no colonies grew from the control vial containing phage without cells, indicating that the phage suspension itself was free of cells. Colonies grew from all the other vials, indicating transduction had occurred. The number of transductants found was proportional to the amount of phage added. The average frequency of transduction in this experiment was 4.1×10^{-5} per PFU.

TABLE 1. *Transduction of B. subtilis 168 (indole$^-$) by phage SP-10 propagated on wild-type strain W-23-S^r**

Cells per ml	Phage particles per ml	Transductants per ml	Transductants per PFU
1.3×10^9	0	0	
1.3×10^9	7.5×10^8	2.7×10^4	3.6×10^{-5}
1.3×10^9	8×10^7	3.7×10^3	4.6×10^{-5}
1.3×10^9	4×10^7	1.9×10^3	4.8×10^{-5}
1.3×10^9	2×10^7	7.3×10^2	3.7×10^{-5}
1.3×10^9	1×10^7	3.6×10^2	3.6×10^{-5}
1.3×10^9	5×10^6	2.1×10^2	4.2×10^{-5}
0	8×10^7	0	

* The cells and phage in a final volume of 1 ml were incubated for 45 min at 37 C and plated on minimal agar.

TABLE 2. *Inhibition of plaque formation by phage antiserum**

Dilution of phage	Plaques per plate	
	With pre-immune serum	With antiserum
10^{-2}	>1000	0
10^{-3}	>1000	0
10^{-4}	>1000	0
10^{-5}	171	0
10^{-6}	16	0

* One ml of pre-immune serum or antiserum plus 0.1 ml SP-10 phage suspension was held 30 min at 25 C. The samples were then diluted in peptone diluent and plated with spores of *B. subtilis* W-23-S^r.

Inhibition of transduction by phage antiserum. Since DNA isolated from wild-type *B. subtilis* has the capacity to transform strain 168 (Spizizen, 1958), I did several experiments to determine whether my observations of change from auxotrophy to prototrophy were in fact a result of transduction or whether DNA present in phage lysates was transforming the cells.

Antiserum capable of combining with phage, as evidenced by its capacity to inhibit plaque formation, was tested for its effect on the ability of phage lysates to transform or transduce cells. Table 2 demonstrates that the antiserum was active in inhibiting plaque formation, and that pre-immune serum had no effect. Table 3 shows the results of an experiment testing the effect of the same antiserum on the transducing system. In addition to strain 168, mutants of W-23-S^r, M1 (arginine$^-$), and M4 (histidine$^-$) were included. Results with all three mutants were similar. Control vials without phage produced no colonies, indicating that none of the cells reverted. Colonies were produced when the vials contained cells, phage, and either peptone or pre-immune serum. No colonies were produced when antiserum was substituted for pre-immune serum. These results indicate that phage is responsible for the genetic change.

Test for effect of deoxyribonuclease on the transducing system. Since transforming DNA is

TABLE 3. *Inhibition of transduction by phage antiserum**

SP-10 phage	Peptone (1%)	Normal serum	Phage antiserum	Transductants per ml		
				Strain 168 (indole$^-$)	Mutant W-23-S^r M1 (arg$^-$)	Mutant W-23-S^r-M4 (his$^-$)
ml	*ml*	*ml*	*ml*			
0	1.5	0	0	0	0	0
0.5	1.0	0	0	3.8×10^3	5.7×10^2	7.4×10^2
0.5	0	1.0	0	3.0×10^3	5.8×10^2	7.7×10^2
0.5	0	0	1.0	0	0	0

* Cells (0.5 ml of 5-hr culture) of the three mutants of *B. subtilis* were incubated 45 min with the constituents as given in the table and samples were plated on minimal agar. SP-10 phage (1×10^9 PFU/ml) was propagated on wild-type strain W-23-S^r.

inactivated by incubation with deoxyribonuclease, an experiment was done to determine whether the enzyme had any effect on the transducing system. The transforming and transducing systems were included in the same experiment to avoid any variations in the recipient cells and the solution of deoxyribonuclease. Table 4 shows the results obtained with the transforming system. The DNA preparation was active in transforming cells of the recipient strain 168, and its activity was destroyed by incubation with deoxyribonuclease. $MgSO_4$ was included in the reaction mixtures to assure activity of the enzyme.

Table 5 shows the results obtained with deoxyribonuclease added to the transducing system. In addition to strain 168, cells of the histidineless mutant, W-23-S^r-M4, were used as recipients. Two phage preparations were also tested. Preparation A was a filtrate from a lysogenic transductant derived from W-23-S^r-M4, and preparation B was a filtrate from a lysogenic strain, W-23-S^r-L9, derived from strain W-23-S^r. Both of the phage preparations were active in transduction, and incubation of the phage preparations with deoxyribonuclease and $MgSO_4$ had no effect on their activities. These results are evidence that the observed genetic changes are mediated by phage, rather than by free DNA that might be present in phage lysates.

TABLE 4. *Effect of deoxyribonuclease on transformation of Bacillus subtilis 168 (indole$^-$)**

Cells	0.05 M $MgSO_4$	0.15 M NaCl	DNA	Deoxyribonuclease (2 mg/ml)	Transformants/ml
	ml	*ml*	*ml*	*ml*	
7×10^8	0.1	0.4			0
7×10^8	0.1	0.3	0.1		2×10^3
7×10^8	0.1	0.2	0.1	0.1	0

* DNA was prepared from wild-type strain W-23-S^r. The reactants (without cells) were incubated 30 min at 37 C. Cells (0.5 ml broth culture) were added, incubation was continued 45 min, and samples were plated on minimal agar.

General transduction. Further studies showed that phage SP-10 participated in general transduction, as indicated in Table 6. Results with the following auxotrophs are shown: 168 (indole$^-$), W-23-S^r-M1 (arginine$^-$), W-23-S^r-M4 (histidine$^-$), and W-23-S^r-M7 (adenine$^-$). Phage propagated on strain W-23-S^r had transducing capacity for each of the four markers. Similarly, phage propagated on an auxotroph transduced the other auxotrophs but not the one on which it was propagated. Additional mutants tested thus far include auxotrophs requiring guanine, thiamine, leucine, or methionine. Each of these was transduced by phage SP-10 propagated on strain W-23-S^r. SP-10 obtained by propagation on wild-type *B. licheniformis* was not tested for transduction of *B. licheniformis* mutants, but it did not transduce strains 168 or W-23-S^r-M4. Similarly,

TABLE 5. *Test for effect of deoxyribonuclease on transduction of Bacillus subtilis**

Recipient cell strain	No. of cells/ml	0.05 M $MgSO_4$	1% Peptone	Phage A	Phage B	Deoxyribonuclease (2 mg/ml)	Transductants/ml
		ml	*ml*	*ml*	*ml*	*ml*	
168 (indole$^-$)	5.6×10^8	0.1	0.5				0
168 (indole$^-$)	5.6×10^8	0.1	0.1	0.4			1.3×10^4
168 (indole$^-$)	5.6×10^8	0.1		0.4		0.1	1.4×10^4
168 (indole$^-$)	5.6×10^8	0.1	0.1		0.4		9.0×10^3
168 (indole$^-$)	5.6×10^8	0.1			0.4	0.1	8.9×10^3
W-23-S^r-M4(his$^-$)	2×10^8	0.1	0.5				0
W-23-S^r-M4(his$^-$)	2×10^8	0.1	0.1	0.4			2.7×10^2
W-23-S^r-M4(his$^-$)	2×10^8	0.1		0.4		0.1	3.2×10^2

* The reactants (without cells) were incubated 30 min at 37 C. Cells (0.4 ml broth culture) were added, incubation was continued for 45 min, and samples were plated on minimal agar. Phage A was a filtrate from a lysogenic transductant of strain W-23-S^r-M4 containing 5×10^8 PFU/ml. Phage B was a filtrate from a lysogenic W-23-S^r strain containing 1×10^9 PFU/ml.

TABLE 6. *General transduction in Bacillus subtilis*

Strain for phage propagation	Recipient strain	Transduction*
W-23-S^r, wild-type	168, indole$^-$	+
	W-23-S^r-M1, arg$^-$	+
	W-23-S^r-M4, his$^-$	+
	W-23-S^r-M7, ad$^-$	+
W-23-S^r-M1, arg$^-$	168, indole$^-$	+
	W-23-S^r-M1, arg$^-$	−
	W-23-S^r-M4, his$^-$	+
	W-23-S^r-M7, ad$^-$	+
W-23-S^r-M4, his$^-$	168, indole$^-$	+
	W-23-S^r-M1, arg$^-$	+
	W-23-S^r-M4, his$^-$	−
	W-23-S^r-M7, ad$^-$	+
W-23-S^r-M7, ad$^-$	168, indole$^-$	+
	W-23-S^r-M1, arg$^-$	+
	W-23-S^r-M4, his$^-$	+
	W-23-S^r-M7, ad$^-$	−

* Plus indicates that transduction occurred and minus indicates that transduction did not occur.

phage SP-10 propagated on *B. subtilis* W-23-S^r did not transduce a lysineless mutant of *B. licheniformis*. Thus far, only a limited number of experiments on transduction between the two species have been done.

Lysogenization of B. subtilis strains. Lysogenic cultures of strain W-23-S^r were isolated from cells grown in the presence of SP-10 phage in NBY broth. Such cultures were immune to lysis by added SP-10 phage, and they caused the indicator strain W-23-S^r to lyse in a cross-streak test. When the lysogenic cultures were grown on potato-extract medium, they sporulated well ($> 1 \times 10^9$ spores per ml) and filtrates usually contained $< 10^3$ PFU per ml. The numbers of viable spores and PFU in the spore suspensions, after heating 1 hr at 65 C, agreed closely, indicating that most, if not all, of the spores were lysogenic. Control experiments showed that free phage was inactivated by the heat treatment. The lysogenic condition of these cultures was maintained through repeated cycles of growth (in potato-extract medium), sporulation, and heat treatment. Similar results were obtained with lysogenic transductants of W-23-S^r auxotrophs.

Strain 168, unlike strain W-23-S^r and its auxotrophs, was insensitive with respect to lysis by phage SP-10 and therefore could not be used as an indicator strain, although cells of strain 168 were recipients in transduction. Neither lytic activity nor propagation of the phage on cells of 168 could be demonstrated, although many tests were run under various conditions. Mutants of phage SP-10 which were virulent for strain W-23-S^r were without any demonstrable lytic activity for strain 168. Lysogeny could not be detected in transductants of strain 168, although a large proportion of transductants derived from auxotrophs of W-23-S^r were lysogenic.

Phage production by lysogenic strains. In contrast to the results obtained when lysogenic cultures were grown in potato-extract medium, poor yields of cells and high yields of phage were produced in media less conducive to sporulation. Three media, minimal broth with yeast extract (3 g/liter), NBY broth, and NBYG broth, were tested for phage production by three lysogenic strains. W-23-S^{r}L1, W-23-S^r-L9, and W-23-S^r-L14 were lysogenized with SP-10 phage. Flasks (250-ml Erlenmeyer) containing 50 ml of medium were inoculated with about 1×10^7 spores and incubated on a shaker at 37 C for 18 hr. The cultures were centrifuged and filtered through fritted glass ultrafine filters to remove bacteria, and were then assayed for PFU against spores of W-23-S^r. The results (Table 7) with the three strains were very similar. The best yields ($> 1 \times 10^9$/ml) were obtained in NBYG broth, and the poorest yields were obtained on the minimal broth with yeast extract.

In further experiments with W-23-S^r-L9 in NBYG broth, phage yields of 1×10^8 to 3×10^8

TABLE 7. *Phage production by three lysogenic strains in three media*

Strain	Medium	PFU/ml
W-23-S^r-L1	Minimal + yeast extract	2.6×10^7
	NBY	1.4×10^9
	NBY + glucose	4.9×10^9
W-23-S^r-L9	Minimal + yeast extract	2.6×10^7
	NBY	1.3×10^9
	NBY + glucose	2.7×10^9
W-23-S^r-L14	Minimal + yeast extract	7.0×10^6
	NBY	3.9×10^8
	NBY + glucose	1.5×10^9

PFU/ml were obtained in 4 hr, and maximal yields of 1×10^9 to 5×10^9 PFU/ml were obtained in 12 to 16 hr.

Stability of SP-10 phage. Preparations of SP-10 propagated on W-23-S^r, or preparations of phage released by strains lysogenic for SP-10, were unstable when stored at 5 C. When phage was propagated by the soft agar layer technique, it was usually harvested in peptone diluent. Such preparations were unstable when stored at 5 C, as evidenced by a rapid decrease in PFU. For example, the titer of a preparation was 1.5×10^9 PFU/ml when harvested, but after 8 days at 5 C the titer had dropped to 5.7×10^8 PFU/ml. Freezing the phage in peptone also resulted in a greatly reduced titer.

Collecting the phage by centrifugation and washing with a solution containing 0.001 M $MgSO_4$ and 0.15 M NaCl (Mg-saline) did not appear to improve the stability of the phage when stored at 5 C in a variety of solutions, including peptone, Mg-saline, minimal medium, and phosphate buffer at pH 7.

Phage preparations were quite stable when stored at −15 C after the addition of 15% (v/v) glycerol. A preparation that was washed twice with Mg-saline and resuspended in a solution containing 0.05 M phosphate (pH 7), 0.15 M NaCl, 0.001 M $MgSO_4$, 0.5% gelatin, and 15% (v/v) glycerol showed no change in plaque count after 2 months at −15 C. It was not necessary to wash the phage preparations to maintain viability by freezing with glycerol. Phage titers of culture filtrates of lysogenic strains were maintained satisfactorily by storage at −15 C after addition of glycerol.

DISCUSSION

By means of transformation, Nester and Lederberg (1961) and Ephrati-Elizur et al. (1961) studied linkage of genetic units controlling the biosynthesis of histidine and tryptophan in *B. subtilis*. Transduction provides another method for studying linkage groups, and the availability of a transducing phage for *B. subtilis* makes it possible for the first time to apply both of these systems to the same strain. Such studies would provide an opportunity for comparing the results obtained by the two methods.

The behavior of the lysogenic cultures grown in various media will be studied further. The observations that they sporulate well and produce very little phage in potato-extract medium, and that in NBYG medium they produce high yields of phage and few, if any, spores, suggest a physiological relationship between phage production and sporulation. Until further studies are done, however, one can not conclude whether such a relationship is apparent or real. In potato-extract medium the lytic cycle of the phage is apparently repressed, permitting the cells to survive and sporulate. But whether this repression is related directly to the process of sporulation can not be determined from the present experiments.

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